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14. ABSTRACT

We have developed a laboratory bio-fuel cell to measure bacterial electrogensis and to select microorganisms that can more efficiently generate electricity from organic sources. Futher optimization of this fuel cell system should allow us to practically implement electric power production from the carbohydrate energy sources that are available in a variety of settings, including littoral zones.

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Efficient Transduction of Electricity by Bacteria

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OBJECTIVES

We have developed a laboratory bio-fuel cell to measure bacterial eletrogenesis and to select microorganisms that can more efficiently generate electricity from organic sources. Further optimization of this fuel cell system should allow us to practically implement electric power production from the carbohydrate energy sources that are available in a variety of settings, including littoral zones.

APPROACH

We employed Shewanella *Oneidensis* MR-1, a facultative anaerobe, to proof and optimize the fuel cell system. This microorganism can use the electrode as an electron acceptor; serial dilution should result in enrichment of bacterial variants that can more efficiently use the electrode for growth. Growth and selection can also be performed using batch culture methods with soluble (fumarate) or insoluble (FeOOH) electron acceptors. Following selection, an enriched microbial population can be diluted into a fresh amperometric poised potential culture system to investigate the efficiency of electricity generation relative to the parental microbes. In the future, we hope to test the hypothesis that deletion or inactivation of nonessential genes can lead to an increase in electrons transferred to the electrode. Libraries of knockout mutants will be generated using random Group II intron insertion and the Mariner transposon Himari I.

ACOMPLISHMENTS

Systems
S. *Oneidensis* MR-1 (ATCC 700550) was grown aerobically in Luria-Bertani (LB) medium, or anaerobically in either LML medium (0.02% yeast, 0.01% peptone, 10mM HEPES, pH 7.4) or growth medium (50 mM sodium phosphate buffer at pH 7.4, 0.1 M NaCl, 1% vitamin mix (ATCC, MD-SD), 1% trace mineral mix (ATCC, MD-TMS)). Incubations were carried at 30°C.

In collaboration with L. Tender (NRL), we have built a two chamber fuel cell as shown in Figure 1. The chambers were
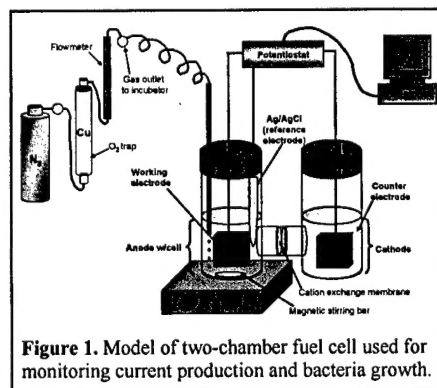

Figure 1. Model of two-chamber fuel cell used for monitoring current production and bacteria growth.

separated with a cation-selective membrane (Nafion 117), and graphite plates (grade G10) were used as electrodes. Electrode connections and pre-treatment were carried out according to established protocols.[1] The liquid volume in each chamber was either 150 or 20mL, based on electrode surface area (70cm^2 or 13cm^2, respectively). The anode chamber was filled with sterilized growth medium and lactate. The cathode chamber was filled with growth medium and aerated. The bacteria for the electrogenesis measurement were inoculated into the anode compartment after the anode was purged with oxygen-free nitrogen. The potential at the anode was maintained against an Ag/AgCl reference electrode (c-905, Electrolytica) with a potentiostat (Model 2053, AMEL Instruments). Current production was monitored through the potentiostat and data was logged with Chart 4.0 software. During bacterial growth, aliquots were taken and the cell density (OD) was measured at 600nm using a UV spectrometer (UV-1601, Shimadzu).

The electrochemical activity of S. *Oneidensis* MR-1 was examined by two methods: 1) Cyclic voltametry of anaerobic cell suspensions was performed using a potentiostat (CH Instrument 832) at 25°C with a scanning rate of 0.05 Vs^{-1}. Bacterial cells grown anaerobically in a serum bottle with LB were washed under strict anaerobic conditions and suspended in phosphate buffer (50 mM, pH 7.0) with 0.1 M NaCl. A 3 mm diameter glassy carbon disc working electrode (MF-2012, BAS), a platinum wire counterelectrode (MW-4130, BAS), and an Ag/AgCl reference electrode (MF-2052, 3M KCl) were used in the electrochemical cell with a working volume of 1mL (see, for example, Figure 4). 2) a batch test was done with amophorous Fe(III) oxyhydroxide (FeOOH; prepared as described by Schwertmann and Cornell[2]) as an electron acceptor. Sterilized serum bottles containing 20 mL of growth medium and 800 µL of saturated FeOOH suspension were prepared by sealing the bottles with butyl rubber stoppers and aluminum caps, and then purging the bottles with deoxygenated N$_2$ for 30 min prior to use. A 1% (v/v) inoculum of an aerobically grown cell culture was added to the serum bottle and further incubated (see, for example, Figure 5).

Serial dilution and batch culture evolution were performed by using sterilized serum bottles containing 20 mL LML medium with 0.2 M lactate and 10 mM fumarate, as described above. To start a series of transfers, the first batch was inoculated with 100 µl of aerobically grown cells by using a sterilized syringe. For subsequent generations, 10 % (v/v) inocula of the previous culture were incubated in a freshly prepared serum bottle containing LML medium, 0.2 M lactate and 10 mM fumarate.

Fuel cell methods
We have previously reported that using our laboratory fuel cell system we could produce electricity from E *Coli* if a soluble mediator was present, or from S. *Oneidensis* MR-1 without a mediator. We have focused on improving this fuel cell system for efficient electric power generation using S. *Oneidensis* MR-1, since this bacterium can directly transfer electrons to the electrode, and thus may be more useful for field applications. Electrogenesis and microbe enrichment were investigated as a function of the poised potential and concentration of lactate.

The effect of poised potential is summarized in Figure 2. Excess lactate (0.5 M) was provided to avoid electron donor limitation. Investigation of the current profile as a function of poised potential (Figure 2A) revealed variable lag periods prior to electrogenesis. These lag times likely

correspond to the reactivation period for aerobically grown cell inocula, as previously reported by Kim et al.[3] <u>We find that electrochemical reactivation of aerobically grown cells can be accelerated by increasing the poised potential; this finding may have significance in terms of recruiting natural bacteria to electrodes newly introduced in the field.</u> Comparing total current (Coulomb = current x

Figure 2. The effect of poised potential on the current generation of S. *Oneidensis* MR-1 in two chambered fuel cell system with 0.5 M lactate. (A) Current profile as a function of time. (B) Total current production(C), rate of electrogenesis and peak current as a function of poised potential.

time) and peak current, total current increased (x 1.06) and peak current decreased (x1.12) slightly as the poised potential was increased (Figure 2B). However, the <u>rate</u> of current production increased dramatically (x6.7) by increasing the poised potential. <u>These results indicate that we can acclerate electrogenesis, and possibly bacterial enrichment, again by using higher poised potentials.</u>
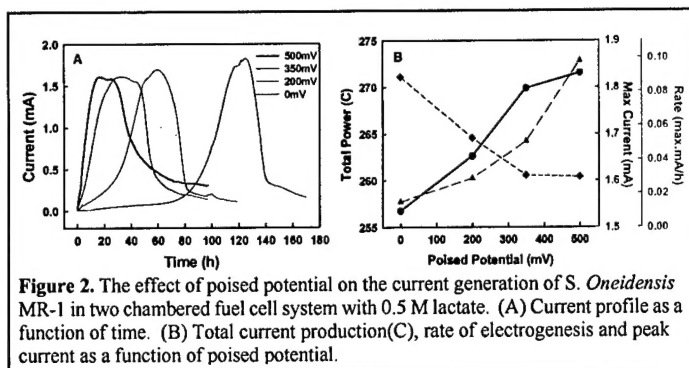
Investigating the effect of fuel (lactate) concentration with graphite electrodes (electrode area = 70 cm^2) poised at 500 mV against Ag/AgCl reference electrode, the current production was both fastest and most efficient at the highest concentration of lactate tested, and became progressively slower and less efficient at decreasing lactate concentrations (Figure 3A). Figure 3B summarizes the effect of fuel

Figure 3. The effect of lactate concentration on the current production of S. *Oneidensis* MR-1 in two chambered fuel cell system with graphite electrode poised at 500 mV. (A) Current profile as a function of time. (B) Total current production(C), rate of electrogenesis and peak current as a function of poised potential.

concentration on the total current production, peak current, and rate of current generation. The total current production (C) and peak current increased dramatically up to ~270 C and ~1.6 mA as lactate concentration increased up to 0.05 M, and then these parameters increased more gradually. The hightest current generation and the hightest peak current with a lactate concentration of 0.5M were 22.6-times and 18.6-times higher, respectively, than the lowest current generation and peak current with a lactate concentration of 0.005M. The rate of current generation increased even more significantly (x84.6) as fuel concentration increased. Overall, S. *Oneidensis* MR-1 electrogenesis is proportional to fuel concentration but can be saturated at 0.05 M lactate. <u>In addition to suggesting that pre-concentration of fueld will allow more efficient electric power in the field, these results suggest that bacterial electrogenesis may be subject to evolutionary improvement (moving the fuel saturation point higher, or 'increasing the V_{max}' for electrogenesis).</u>
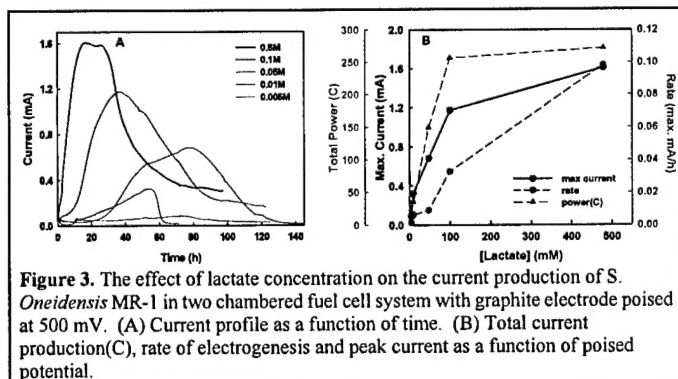
Batch methods
Cyclic voltammograms (CV, Figure 4) of cell suspensions show that S. *Oneidensis* MR-1 cells grown under anaerobic condition have a redox potential of around -0.2 V against Ag/AgCl reference electrode,
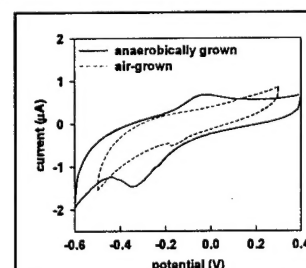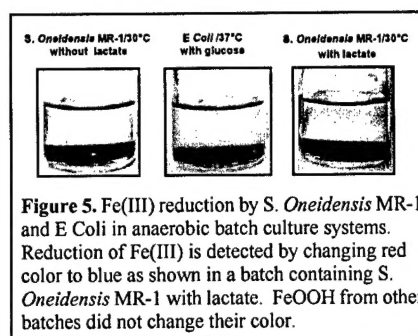

Figure 4. Cyclic Voltammogram of S *Oneidensis* MR-1 grown anaerobic (black solid line) and aerobically (red dotted line).

whereas cells grown under aerobic conditions do not have a measurable redox potential (this data has been presented in a previous report). The ability of anaerobically grown S. *Oneidensis* MR-1 to reduce insoluble Fe (III) to Fe (II) in the presence of fuel (electron donor, lactate) was investigated in liquid cultures supplemented with amophorous iron oxyhydroxide (FeOOH). Two samples were prepared as controls: one had no lactate, while the other had glucose as a fuel source but anaerobically grown E *Coli* was inoculated into the media rather than S. *Oneidensis*

Figure 5. Fe(III) reduction by S. *Oneidensis* MR-1 and E Coli in anaerobic batch culture systems. Reduction of Fe(III) is detected by changing red color to blue as shown in a batch containing S. *Oneidensis* MR-1 with lactate. FeOOH from other batches did not change their color.

MR-1. The color of the FeOOH suspension was investigated after overnight incubation. FeOOH in a batch containing S. *Oneidensis* MR-1 with lactate turned to blue from red, due to the reduction of Fe (III) to Fe(II) as cells grew anaerobically (Figure 5). On the contrary, both controls (without lactate, or with E *Coli* and glucose) did not change the color of FeOOH. Because S. *Oneidensis* MR-1 could not grow without lactate, there was no electron production, and thus Fe (III) was not reduced. Similarly, FeOOH did not change its color in the presence of E *Coli*, although the media became cloudy. This result is consistent with previous reports that E *Coli* cannot shift electrons to insoluble electron acceptors without a mediator.[4] These results confirm that electron exchange is possible between the electrode surface and the anaerobically grown S. *Oneidensis* MR-1 and thus establish the batch culture method for the simple determination of electrochemical activity of bacteria under anaerobic conditions. The utility of this assay should not be underestimated, as it allows robust microbiological screens and selections to be carried out for electrogenesis.

Evolutionary experiments

In initial experiments we did not see significant bacterial enrichment in solution. When the OD of the media was monitored it was found to increase by ≈ 50 % from cell inoculation through the acquisition of peak current, regardless of fuel cell conditions. It is suspected that since S. *Oneidensis* MR-1transfers electrons directly to the anode, like Geobacter sulfurreducens,[5] that it becomes enriched on the electrode surface, rather than in solution.

We then tried serial dilution of bacteria in a batch culture system with insoluble FeOOH as an electron acceptor to enrich bacteria with efficient electricity transduction. However, once bacteria became enriched on the surface of FeOOH (as indicated by the change to blue), they could not be induced to grow further either by dilution of the reduced FeOOH or the media supernatant into fresh media.

Finally, as an alternative enrichment method for the batch culture system we employed fumarate instead FeOOH, since fumarate is known to be a soluble electron acceptor.[6] As summarized in Figure 6A, cell growth was similar in each dilution, in which the maximum OD after 24h incubation was between 0.12 and 0.15. Frozen stocks of enriched cells were made from every 5
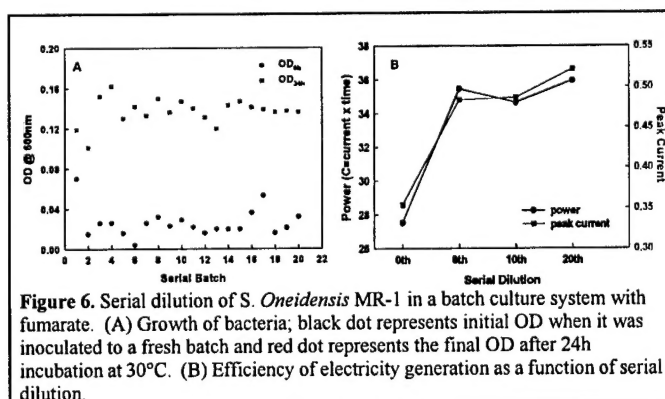

Figure 6. Serial dilution of S. *Oneidensis* MR-1 in a batch culture system with fumarate. (A) Growth of bacteria; black dot represents initial OD when it was inoculated to a fresh batch and red dot represents the final OD after 24h incubation at 30°C. (B) Efficiency of electricity generation as a function of serial dilution.

batches (1st, 5th, 10th, 15th, and 20th) and tested for the efficiency of electricity generation using the fuel cell system (electrode area = $13cm^2$, growing medium volume = 20 mL, frozen cultures were revived in LB medium aerobically.). As summarized in Figure 6B, after 5th dilution bacteria showed better electricity transduction with higher peak current. <u>This result implied that we can enrich and potentially select microbe populations that are more efficient in electric power production using this batch culture system.</u>

It should be noted that the phenomena reported in Figure 6B differs significantly from the recovery from aerobic growth noted previously; this is because the measurements were all taken with cells that had similar physiological histories. Once cells were enriched anaerobically in the batch culture system, frozen stocks were made. Therefore for each point on the graph in Figure 6B, the stored cells were first grown out in LB under aerobic conditions, and were then inoculated into an anaerobic fuel cell at identical cell densities (OD). In addition to having similar histories, no significant difference was seen between generations in the lag time for anaerobic electricity production, again unlike the case where there was recovery from aerobic growth. While it seems unlikely that significant evolution could have occurred over only five generations, it is nonetheless quite plausible that the cells have physiologically adapted to the batch culture conditions, perhaps by producing significantly more surface cytochromes. In addition, the fact that we can select for and see such physiological adaptations implies that it should also be possible to see evolutionary changes as well.

CONCLUSION AND SIGNIFICANCE

We have developed a bacterial fuel cell system and optimized this system for electric power generation. We could generate electricity from S. *Oneidensis* MR-1 without an electron mediator. Increasing the poised potential of the anode increased current generation by 6.7-fold. Increasing lactate concentration resulted in 84.6-fold more current generation, 22.6-fold more power production, and 18.6-fold higher peak current. In addition, we demonstrated that aerobically grown S. *Oneidensis* MR-1 can produce electricity in a fuel cell system via reactivation of electrochemical activity. We believe that modulation of these parameters may also be useful for the optimization of bacterial fuel cells in the field.

We performed serial, batch dilution experiments with bacteria with soluble electron acceptors, and then tested their electron transduction efficiency using a fuel cell system. After 5 serial dilutions, enrichment or physiological alterations resulted in significantly improved efficiency in electricity generation. In addition, we have demonstrated that we can readily assay the electrochemical activity of microbes by using a batch culture system with the insoluble electron acceptor, FeOOH, in which the red color (Fe (III)) was turned to blue (Fe (II)) as microbes produced electricity. These experiments provide the necessary proofs to continue the evolution of electrogenic microorganisms both in solution and on the solid phase.

PATENT INFORMATION

N/A

AWARD INFORMATION

N/A

PUBLICATIONS AND ABSTRACTS

Matsumura, I., Ellington, A.D. In vitro evolution of beta-glucuronidase into a beta-galactosidase proceeds through non-specific intermediates. *Journal of Molecular Biology*, 305(2)331-339, 2001.

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